Cell PRESS
Developmental Cell
Article

# Side-Chain Modification of Cytokinins Controls Shoot Growth in *Arabidopsis*

Takatoshi Kiba,[1] Kentaro Takei,[2] Mikiko Kojima,[1] and Hitoshi Sakakibara[1,*]
[1]RIKEN Center for Sustainable Resource Science, 1-7-22, Suehiro, Tsurumi, Yokohama 230-0045, Japan
[2]RIKEN Plant Science Center, 1-7-22, Suehiro, Tsurumi, Yokohama 230-0045, Japan
*Correspondence: sakaki@riken.jp
http://dx.doi.org/10.1016/j.devcel.2013.10.004

## SUMMARY

**Cytokinins (CKs), a class of plant hormones, are central regulators of plant growth and development. Based on numerous physiological and genetic studies, the quantitative regulation of cytokinin levels is the major mechanism regulating cytokinin action in diverse developmental processes. Here, we identified a different mechanism with which the physiological function of CK is modulated through side-chain modification (*trans*-hydroxylation). The *trans*-hydroxylation that forms *trans*-zeatin (tZ)-type CK from $N^6$-($\Delta^2$-isopentenyl)adenine (iP)-type CK is catalyzed by the cytochrome P450 enzymes CYP735A1 and CYP735A2 in *Arabidopsis*. Deficiency in *trans*-hydroxylation activity results in dramatic retardation of shoot growth without affecting total CK quantity, while augmentation of the activity enhances shoot growth. Application of exogenous tZ but not iP recovers the wild-type phenotype in the mutants, indicating that *trans*-hydroxylation modifies the physiological function of CK. We propose that the control of cytokinin function by side-chain modification is crucial for shoot growth regulation in plants.**

## INTRODUCTION

Cytokinins (CKs) are a class of phytohormones implicated in diverse processes of plant growth and development, including cell division, germination, root and shoot growth, cambial proliferation, stress and nutritional responses, and senescence (Gan and Amasino, 1995; Matsumoto-Kitano et al., 2008; Mok and Mok, 2001; Riefler et al., 2006; Sakakibara, 2006; Werner et al., 2001). Naturally occurring CKs are $N^6$-prenylated adenine derivatives. Although collectively referred to as CKs, there are significant variations in the side-chain structure (Mok and Mok, 2001; Shaw, 1994). The most common derivatives found in higher plants are $N^6$-($\Delta^2$-isopentenyl)adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DZ), which differ in the presence and stereoisomeric position of a hydroxyl group at the end of the prenyl side chain (Mok and Mok, 2001; Sakakibara, 2006). iP and tZ are the major forms in *Arabidopsis thaliana*; they are perceived by three CK receptors, *ARABIDOPSIS* HISTIDINE KINASE 2 (AHK2), AHK3, and AHK4/CRE1 (AHK4; Higuchi et al., 2004; Inoue et al., 2001; Nishimura et al., 2004; Riefler et al., 2006; Suzuki et al., 2001; Yamada et al., 2001).

In the current model of CK biosynthesis in higher plants (Figure S1A available online; for review, see Sakakibara, 2006), the initial step is the $N^6$-prenylation of adenosine 5′-phosphates catalyzed by adenosine phosphate-isopentenyltransferase (IPT) to form iP-riboside 5′-phosphates (iPRPs; Kakimoto, 2001; Takei et al., 2001a). The cytokinin-activating enzyme LONELY GUY (LOG) then converts iP-riboside 5′-monophosphate to iP (Kurakawa et al., 2007; Kuroha et al., 2009; Tokunaga et al., 2012). The key step in tZ biosynthesis is the *trans*-hydroxylation of the prenyl side chain of iPRPs to produce tZ-riboside 5′-phosphates (tZRPs), which are then activated by LOG through transformation into tZ. Cytochrome P450 monooxygenase CYP735A, encoded by *CYP735A1* and *CYP735A2* in *Arabidopsis*, has been shown to catalyze the *trans*-hydroxylation step in vitro (Takei et al., 2004b).

Classical bioassays performed decades ago suggested that the side-chain structure of CK is relevant to its biological activity (Mok et al., 1978; Schmitz and Skoog, 1972). More recently, it has been demonstrated that each CK receptor and CK oxidase (CKX; a class of CK-degrading enzymes) has a different spectrum of side-chain preference (Choi et al., 2012; Galuszka et al., 2007; Inoue et al., 2001; Romanov et al., 2006; Spíchal et al., 2004; Stolz et al., 2011; Yamada et al., 2001; Yonekura-Sakakibara et al., 2004). For instance, in vitro binding assays have shown that AHK2 and AHK4 bind iP as well as tZ with high affinity, while AHK3 has a ten times lower affinity to iP than to tZ (Romanov et al., 2006; Stolz et al., 2011). Furthermore, a difference in side-chain preference between phloem- and xylem-borne CKs has been reported (Corbesier et al., 2003; Hirose et al., 2008; Kudo et al., 2010; Takei et al., 2001a). However, whether side-chain modification of CKs has a regulatory role in plant growth and development remains to be determined.

To elucidate the physiological roles of CK side-chain variation, analyses of mutants impaired in side-chain modification are essential. Here, we identified *Arabidopsis* loss-of-function mutants of *CYP735A1* and *CYP735A2* impaired in the side-chain modification (*trans*-hydroxylation) required to form tZ-type CKs from iP-type CKs, and show that *trans*-hydroxylation modifies the physiological function of the CK. tZ enhances shoot growth, but iP does not. Interestingly, analysis of CK receptor mutants in the *cyp735a1 cyp735a2* double mutant background suggests that the binding affinities of CK receptors might not be the only determinants of the functional differentiation between iP and tZ. We propose that *trans*-hydroxylation of CK confers functional specificity that is required for shoot growth regulation.

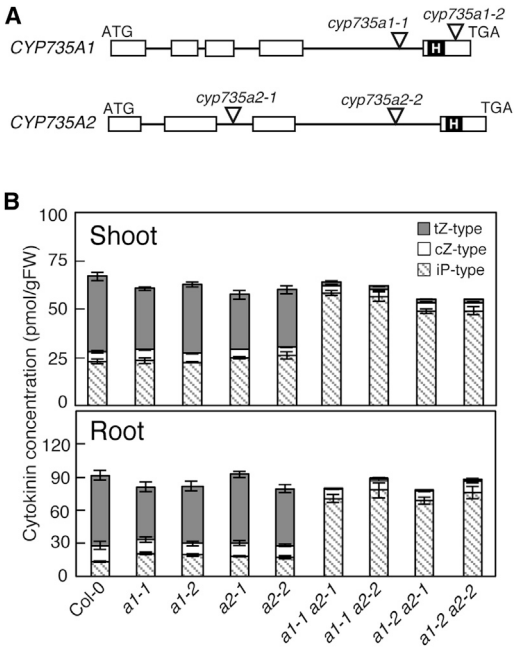



**Figure 1. The *cyp735a1 cyp735a2* Double Mutants Show *trans*-Zeatin-Type Cytokinin Deficiency**

(A) Schematic representation of *cyp735a1* and *cyp735a2* T-DNA insertion alleles. Boxes represent exons; horizontal bars, introns; triangles, T-DNA insertion sites; ATG, initiation codon; TGA, termination codon. The label "H" in the fifth exon represents the region encoding a heme-binding signature, which is essential for P450 catalytic activity.

(B) Cytokinin concentrations in the shoot (upper panel) and root (lower panel) of the wild-type (Col-0), *cyp735a1-1* (*a1-1*), *cyp735a1-2* (*a1-2*), *cyp735a2-1* (*a2-1*), *cyp735a2-2* (*a2-2*), *cyp735a1-1 cyp735a2-1* (*a1-1 a2-1*), *cyp735a1-2 cyp735a2-1* (*a1-2 a2-1*), *cyp735a1-1 cyp735a2-2* (*a1-1 a2-2*), and *cyp735a1-2 cyp735a2-2* (*a1-2 a2-2*). Seedlings were grown for 18 days on MGRL agar plates before shoots and roots were harvested. gFW, gram fresh weight; tZ-type, *trans*-zeatin and its conjugates; cZ-type, *cis*-zeatin and its conjugates; iP-type, $N^6$-($\Delta^2$-isopentenyl)adenine and its conjugates. Error bars represent SD of four biological replicates. The concentration of each cytokinin molecular species in the shoot and root is shown in Table S1.

See also Figure S1.

## RESULTS

### Identification of T-DNA Insertion Mutants of *CYP735A1* and *CYP735A2*

Two independent T-DNA insertion alleles for *CYP735A1* as well as *CYP735A2* were obtained from the *Arabidopsis* Biological Resource Center (Figure 1A), and all four possible double mutants were generated by crossing. Because three of the four T-DNA insertions were located in introns, expression levels of *CYP735A* genes were analyzed with RT-PCR. Full-length transcripts of the corresponding *CYP735A* genes were not detected in any of the single and double mutants even after 40 PCR cycles, which yielded saturating amplification of *CYP735A1* and *CYP735A2* transcripts in wild-type Col-0 plants (Figure S1B). The insertions, except for *cyp735a1-2*, were situated upstream of the region encoding a heme binding signature that appears to be essential for P450 catalytic activity (Figure 1A; Schalk et al., 1999), indicating that truncated proteins produced in these mutants, if any, are inactive. We concluded that *cyp735a1-1*, *cyp735a2-1*, and *cyp735a2-2* were null alleles.

### *CYP735A1* and *CYP735A2* Are Required for tZ-Type Cytokinin Biosynthesis in *Arabidopsis*

First, single and double mutants of *CYP735A1 and CYP735A2* were subjected to hormone analysis to examine the role of *CYP735A*s in CK side-chain modification in *Arabidopsis*. In the single mutants *cyp735a1* and *cyp735a2*, slight decreases in tZ-type CK (tZ and its conjugates) concentrations in the shoot and slight increases in iP-type CK (iP and its conjugates) concentrations in the root were observed as compared to the wild-type (Table S1). In all *cyp735a1 cyp735a2* double-mutant combinations, the concentration of tZ-type CKs diminished to less than 5% of the wild-type levels in shoots and roots, while iP-type CK levels increased more than 2-fold so that the loss of tZ-type CKs was compensated by iP-type CKs. It should be noted that tZ-type CKs did not completely disappear in the mutants (Figure 1B; Table S1). The tZ and tZ-9-*N*-glucoside concentrations were mostly under the detection limit, but other tZ sugar and sugar phosphate conjugates, such as tZ riboside, tZRPs, tZ-*O*-glucoside and tZ-7-*N*-glucoside, were detected at low levels. On the other hand, no consistent changes in the levels of cZ-type CKs, abscisic acid, and indole-3-acetic acid were detected in the single and double mutants compared with the wild-type (Figures 1B, S1C, and S1D; Table S1). We further confirmed that the side-chain profile alteration in *cyp735a1 cyp735a2* could be complemented by the introduction of a genomic fragment containing either *CYP735A1* or *CYP735A2* (Figures S1E and S1F). These results demonstrated that CYP735A1 and CYP735A2 are the major enzymes that catalyze *trans*-hydroxylation of the CK side chains to synthesize tZ-type CKs in *Arabidopsis*.

### The *cyp735a1 cyp735a2* Double Mutants Show Retardation in Shoot Growth and Development

To explore the consequence of altered CK side-chain profiles (CK quality) in plants, single and double mutants of *CYP735A1* and *CYP735A2* were grown on soil, and shoot growth and development were examined at various developmental stages. Although no visible phenotype was found in any of the single mutants, significant alterations of growth and development were observed in the aerial parts of the double mutants (Figures 2 and S2; Table S2). Because all four double-mutant combinations displayed essentially the same hormonal and developmental phenotypes (Figures 1 and S2), we here refer to any of the double mutants as *cyp735a1 cyp735a2* (*cypDM*), unless otherwise indicated.

In the vegetative development of *cypDM*, a retardation of shoot growth was apparent as a significant reduction in rosette diameter (Figures 2A, S2A, and S2B). Bolting time was somewhat delayed but leaf number at bolting was not altered, indicating that the rate of leaf formation is only slightly slower in *cypDM* than in the wild-type (Table S2). The mutant grew rosette leaves with reduced leaf blade size and petiole length (Figure 2B). The leaves of the mutants were rounder than those of the wild-type (Figure 2B) and showed delayed abaxial trichome production (Table S2), implying that the transition of shoot development from the juvenile to the adult phase was delayed. However, dark-induced and age-dependent leaf senescence, determined by

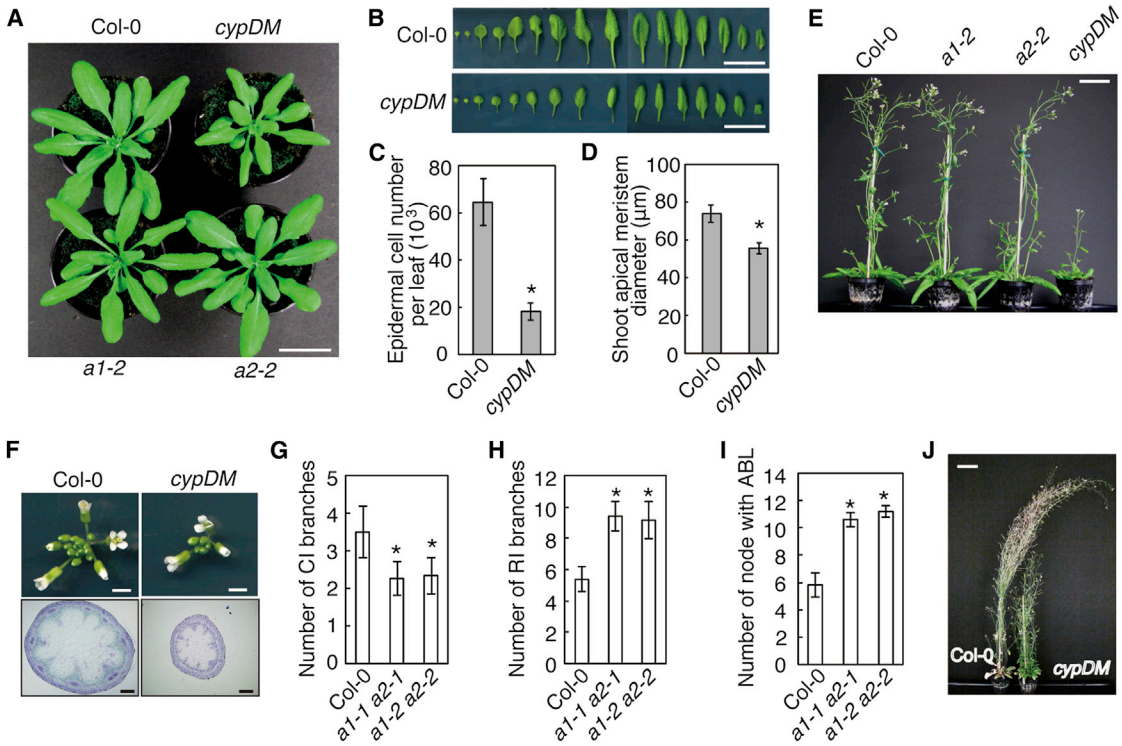



**Figure 2. Vegetative and Reproductive Growth of the Shoot Is Retarded in *cyp735a1 cyp735a2* Mutants**

(A) Appearance of 27-day-old wild-type (Col-0), *a1-2 a2-2* (*cypDM*), *a1-2*, and *a2-2* grown on soil.

(B and C) Rosette leaves (B) and epidermal cell number (C) of 27-day-old Col-0 and *cypDM* grown on soil.

(D) Shoot apical meristem size of 5-day-old Col-0 and *cypDM* seedlings.

(E) Appearance of 37-day-old Col-0, *a1-2*, *a2-2* and *cypDM* grown on soil.

(F) Inflorescences and transverse sections of inflorescence stems of Col-0 and *cypDM* harvested at 37 days after germination.

(G–I) Shoot branching phenotype at 40 days after germination (n > 10). (G) Number of primary cauline branches (CI). (H) Number of primary rosette branches (RI). (I) Number of nodes with axillary bud leaves (ABL).

(J) Terminal phenotype of *cypDM* at 49 days after germination. Scale bars: 2 cm for (A) and (B), 5 cm for (E) and (J), 2 mm for (F, upper panel), and 0.2 mm for (F, lower panel). Error bars represent SD of more than three biological replicates. Asterisks indicate statistically significant differences compared with Col-0 ($p < 0.05$, one-way ANOVA followed by Dunnett's test).

See also Figure S2 and Table S2.

measuring chlorophyll content, was unaffected (Figures S2D and S2E). Close inspection of epidermal cells on mature rosette leaves revealed that cell number per leaf was decreased, suggesting that meristem activity was affected in *cypDM* (Figure 2C). Therefore, shoot apical meristems (SAM) were examined. The SAM diameter of *cypDM* was significantly smaller than that of the wild-type (Figure 2D). In the reproductive stage, *cypDM* developed a short and thin primary inflorescence stem, and formed a reduced number of flower buds and flowers (Figures 2E, 2F, and S2C), suggesting that cambial and inflorescence meristem activities were impaired. After the onset of bolting, it was obvious that shoot branching was enhanced in *cypDM* (Figure 2J). The numbers of nodes with axillary bud leaves and of primary rosette branches were increased, while that of primary cauline branches was decreased (Figures 2G–2I and S2F; Table S2). These morphological phenotypes were complemented by genomic fragments containing either *CYP735A1* or *CYP735A2* (Figure S2G), demonstrating that the loss of CYP735A activity was the cause of the mutant phenotypes.

Except for shoot branching, the phenotypes of *cypDM* resemble those of *ahk2 ahk3* and *ipt3 ipt5 ipt7* (*ipt357*) mutants (Higuchi et al., 2004; Miyawaki et al., 2006; Nishimura et al., 2004; Riefler et al., 2006), which have reduced CK activities due to diminished CK signaling activities and CK levels, respectively. Taken together, these findings suggest that *cypDM* has a reduced CK activity in the shoot.

## The Effects of *cyp735a1 cyp735a2* Mutations Are Stronger in Shoots Than in Roots

Reductions in CK activity generally result in an enhancement of root growth (Argyros et al., 2008; Dello Ioio et al., 2007; Higuchi et al., 2004; Miyawaki et al., 2006; Nishimura et al., 2004; Riefler et al., 2006; Werner et al., 2001, 2003; Yokoyama et al., 2007). To assess whether root growth is affected in *cypDM*, wild-type and *cypDM* plants were grown in hydroponic culture. The shoot biomass was reduced significantly in *cypDM* shoots but not in roots (Figures 3A and 3B). Furthermore, no differences were observed between wild-type and *cypDM* roots with respect to cellular organization around columella meristem and vasculature where *CYP735A2* is highly expressed (Figures S3 and S5). To examine whether CK was involved in the development of this phenotypic difference between shoots and roots, we analyzed

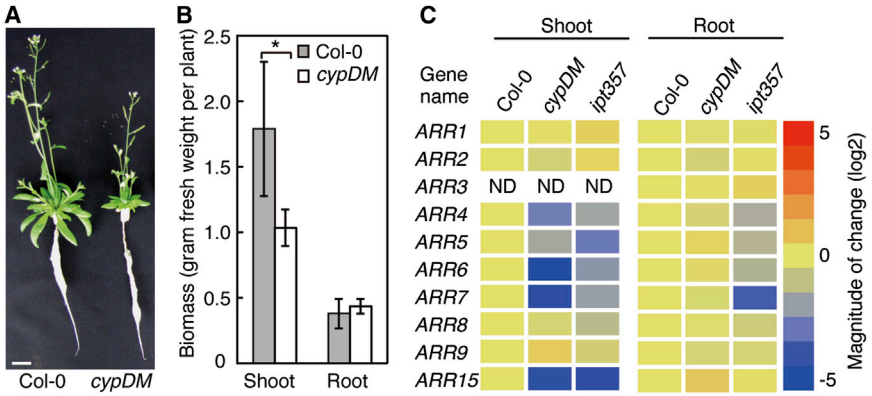



**Figure 3. The *cyp735a1 cyp735a2* Mutant Shows a More Pronounced Phenotype in the Shoot Than in the Root**

(A and B) Overall stature (A) and biomass (B) of 28-day-old hydroponically grown wild-type (Col-0) and *a1-2 a2-2* (*cypDM*). Error bars represent SD of four biological replicates. The asterisk indicates a statistically significant difference compared with Col-0 ($p < 0.05$, Student's t test). Scale bar: 2 cm.

(C) Expression analysis of cytokinin-inducible type-A *ARR* genes (*ARR3-9*, *ARR15*) and non-cytokinin-responsive type-B *ARR* genes (*ARR1*, *ARR2*) in the shoot and root of Col-0, *cypDM*, and *ipt3 ipt5 ipt7* (*ipt357*). The magnitudes of the changes in transcript levels relative to Col-0 are color-coded. Numeric expression values are shown in Table S3. ND, not detected.

See also Figure S3.

CK activity by comparing the expression levels of immediate-early CK-inducible type-A *ARR*s (*ARR3-9*, *15*) using quantitative RT-PCR analysis (qPCR). The *ipt357* mutant was used as a typical mutant showing low CK activity in both shoot and root due to reduced CK quantities (less than 20% of the wild-type levels of iP- and tZ-type CKs; Miyawaki et al., 2006). In the shoot, *cypDM* and *ipt357* showed significantly decreased expression of many type-A *ARR*s, while the expression of non-CK-inducible type-B *ARR*s (*ARR1* and *ARR2*) was not dramatically changed (Figure 3C; Table S3). Thus, CK activities in *cypDM* and *ipt357* shoots probably are lower than in wild-type shoots. Although similar reductions in CK activity were observed in *ipt357* roots, the activity in *cypDM* roots was comparable to that in wild-type roots (Figure 3C; Table S3). These results supported the interpretation that the *cypDM* phenotype was more pronounced in shoots than in roots due to decreased CK activities in the shoots, and showed that changes in CK quality and quantity resulted in distinct phenotypes.

## The Growth Phenotype of *cyp735a1 cyp735a2* Is Rescued by tZ but Not by iP

Next, we examined whether the growth retardation of *cypDM* was caused by a decrease in the level of CKs with certain side-chain structures. We fed *cypDM* with CK bases known to be active in *Arabidopsis* (iP, tZ, and DZ) by spraying. As shown in Figure 4, application of tZ to *cypDM* at 1 μM completely recovered rosette diameter and partially recovered inflorescence stem length. The bushy appearance of *cypDM* also was suppressed by the tZ-treatment (Figure 4A). Although maximal recovery was observed when tZ was applied at 1 μM, iP and DZ at this concentration did not rescue the phenotype (Figure 4). iP did not prevent the development of the *cypDM* phenotype even at 10 μM (Figure 4), indicating that iP and tZ have distinct physiological functions. It should be added that application of iP to the wild-type did not result in a *cypDM*-like phenotype (data not shown). Collectively, these data strongly suggest that the *cypDM* phenotype is caused by a deficiency of tZ-specific functions.

## Increased tZ-Type CK Levels Enhance Shoot Growth

To further understand the role of tZ in shoot growth, transgenic *Arabidopsis* plants overexpressing *CYP735A2* under the control of the cauliflower mosaic virus 35S promoter (*CYP735A2-ox*) were generated. Enhanced accumulation of *CYP735A2* mRNA in the shoots of two independent lines of *CYP735A2-ox* was confirmed by qPCR (Figure 5A). In the shoots of these transgenic plants, the total CK concentration did not differ significantly from that in the wild-type (Figure 5B; Table S4). However, a proportional increase of tZ-type CKs with a concomitant decrease of iP-type CKs resulted in transgenic plants whose CKs were mostly of the tZ-type (Figure 5B; Table S4). When grown on soil, these transgenic plants developed a rosette with significantly greater diameter and fresh weight than the wild-type (Figures 5C and 5D), demonstrating that an increase in tZ-type CK concentration is sufficient to enhance shoot growth. In the roots of these overexpressors, similar proportional increases of tZ-type CKs and small increments in total CK concentration were observed (Figure S4C; Table S4). However, root growth was not reduced in the overexpressors (Figures S4A and S4B). These results indicated that tZ acts as a positive regulator of shoot growth.

## All CK Receptors Play a Role in tZ Perception for Shoot Growth

Previous studies have shown that each CK receptor (AHK2, AHK3, and AHK4) in *Arabidopsis* has different ligand affinities toward iP and tZ (Romanov et al., 2006; Spíchal et al., 2004; Stolz et al., 2011). To determine the CK receptor relevant for tZ perception in shoot growth, we evaluated the effects of tZ-deficiency on mutants possessing only one CK receptor gene. The mutants *ahk2 ahk3*, *ahk3 ahk4,* and *ahk2 ahk4* express only *AHK4*, *AHK2*, and *AHK3*, respectively. We crossed these mutants with *cypDM* to generate *ahk2 ahk3 cypDM*, *ahk3 ahk4 cypDM*, and *ahk2 ahk4 cypDM* quadruple mutants, and shoot growth was analyzed.

As has been reported previously, *ahk2 ahk3* plants exhibit a severe reduction in shoot growth compared with the wild-type, whereas *ahk2 ahk4* do not show this effect (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Under our culture conditions, a small but significant decrease in rosette diameter (−13%) and dry weight (−22%) was detected in *ahk3 ahk4* plants (Figures 6A and 6B). When grown on soil, each quadruple mutant developed a smaller rosette than the parental *ahk* double mutant and *cypDM* (Figures 6A and 6B). Detailed analysis

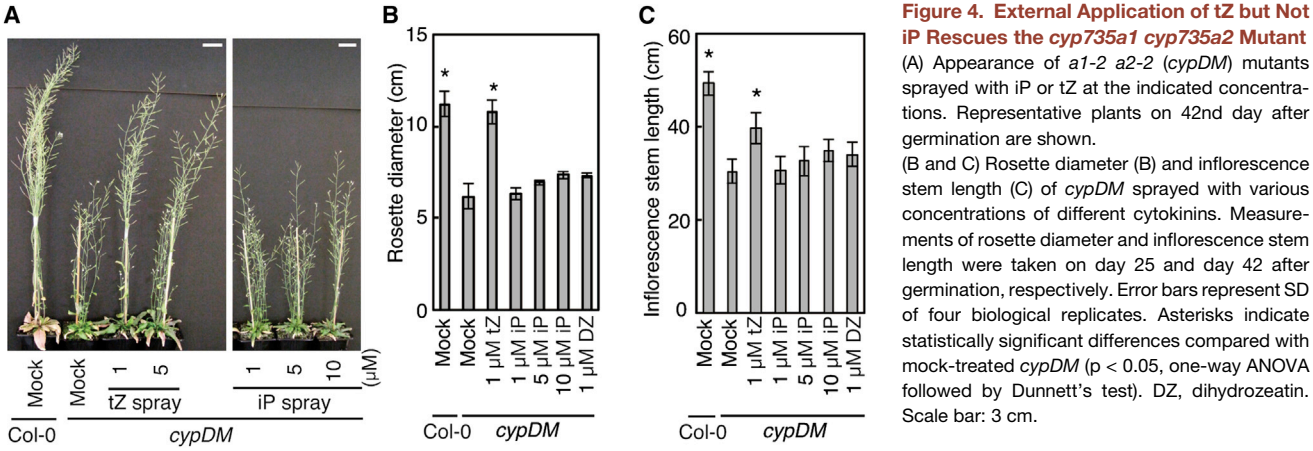



**Figure 4. External Application of tZ but Not iP Rescues the *cyp735a1 cyp735a2* Mutant**

(A) Appearance of *a1-2 a2-2* (*cypDM*) mutants sprayed with iP or tZ at the indicated concentrations. Representative plants on 42nd day after germination are shown.

(B and C) Rosette diameter (B) and inflorescence stem length (C) of *cypDM* sprayed with various concentrations of different cytokinins. Measurements of rosette diameter and inflorescence stem length were taken on day 25 and day 42 after germination, respectively. Error bars represent SD of four biological replicates. Asterisks indicate statistically significant differences compared with mock-treated *cypDM* ($p < 0.05$, one-way ANOVA followed by Dunnett's test). DZ, dihydrozeatin. Scale bar: 3 cm.

revealed that the effect of *cyp735a1 cyp735a2* double mutations was more severe in *ahk2 ahk4* than in wild-type, *ahk2 ahk3*, and *ahk3 ahk4* (Figure 6C). This indicated that while all CK receptors contributed to tZ perception for shoot growth, AHK3 played a slightly more important role than the others. Because AHK2 and AHK4 have equally high affinities to iP and tZ (Inoue et al., 2001; Romanov et al., 2006; Spíchal et al., 2004; Stolz et al., 2011; Yamada et al., 2001), we expected that any decrease in AHK2- or AHK4-mediated CK activity caused by a decreased level of tZ-type CKs would be completely compensated by a corresponding increase in iP-type CKs (Figure 1B). Surprisingly, the effects of the *cyp735a1 cyp735a2* double mutation were evident in the *ahk3 ahk4* and *ahk2 ahk3* background (Figure 6). Thus, the ligand preferences of the CK receptors do not seem to be the only determinants of the functional differences between iP and tZ in the shoot.

### *CYP735A* Genes Are Predominantly Expressed in the Root Vascular Bundle

The *cypDM* phenotype indicated that *CYP735A1* and *CYP735A2* are required for shoot growth and development (Figures 2 and S2). However, qPCR analysis showed that transcripts of the *CYP735A*s were more abundant in roots than in shoots (Takei et al., 2004b). To determine how the expression patterns of the *CYP735A*s correlate with the *cypDM* phenotype, we analyzed the tissue specificity of the *CYP735A1* and *CYP735A2* promoters by GUS staining. We generated transgenic *Arabidopsis* carrying *CYP735A* promoter:beta-glucuronidase transcriptional fusion genes (ProCYP735A1:GUS and ProCYP735A2:GUS). In ProCYP735A1:GUS plants, no GUS activity was detected, which appeared in line with the low expression levels of *CYP735A1* in all organs that have already been described (Takei et al., 2004b). In ProCYP735A2:GUS plants, GUS staining was strong in the root but much weaker in the hypocotyl and shoot apical region, again in agreement with Takei et al. (2004b); see Figures S5A–S5C. In the root apex of primary roots, staining was observed in columella cells but not in meristematic cells (Figure S5D). In other parts of primary and lateral roots, *CYP735A2* expression was restricted to the vascular bundle. The expression was undetectable in the elongation zone and differentiation zone but appeared eventually with maturation (Figure S5A), although it declined again with age (Figures S5B and S5E–S5G). Transverse sections of the stained primary root revealed strong expression within the entire vascular bundle, including pericycle, phloem, and procambium cells (Figure S5H).

### Root-Derived tZ-Type Cytokinins Are Transported to Regulate Shoot Growth

CKs may act as long-distance regulatory signals because they are found in the phloem and xylem sap (Corbesier et al., 2003; Hirose et al., 2008; Kudo et al., 2010; Matsumoto-Kitano et al., 2008; Takei et al., 2001b). The discrepancy between the phenotypically affected shoot organs and the location of *CYP735A* expression in the roots prompted us to test whether root-borne tZ-type CK can be transported to regulate shoot growth. To test this idea, wild-type and *cypDM* were intergrafted to produce chimeric plants consisting of a *cypDM* shoot scion on a wild-type root stock (*cypDM*/WT), or vice versa (WT/*cypDM*). In the *cypDM*/WT graft, rosettes and inflorescence stems of *cypDM*-derived shoots grew as large as in the wild-type (Figures 7A and 7B). CK analysis revealed that tZ-type CK concentration and CK composition in *cypDM*-derived shoots recovered to wild-type levels (Figure 7C; Table S5), indicating that root-borne tZ-type CKs are sufficient to maintain CK levels and growth in the shoot. On the other hand, neither growth retardation nor dramatic changes in CK concentration and composition were observed in shoots of WT/*cypDM* grafts (Figure 7; Table S5), indicating that the shoot itself can compensate for the loss of root-borne tZ-type CKs. Such homeostatic mechanisms for regulation of shoot CK levels have been described (Foo et al., 2007). These results suggested that root-borne tZ-type CKs have a potential to regulate shoot growth though they are not essential for normal shoot growth.

## DISCUSSION

Numerous physiological and genetic studies have shown that CK action in plants is controlled by CK quantity (Bartrina et al., 2011; Gan and Amasino, 1995; Kurakawa et al., 2007; Kuroha et al., 2009; Miyawaki et al., 2006; Mok and Mok, 2001; Mok, 1994; Nieminen et al., 2008; Takei et al., 2001b; Tokunaga et al., 2012; Walch-Liu et al., 2000; Werner et al., 2001, 2010). Here, we identified a different mechanism that the physiological function of CK is modulated by chemical transformations

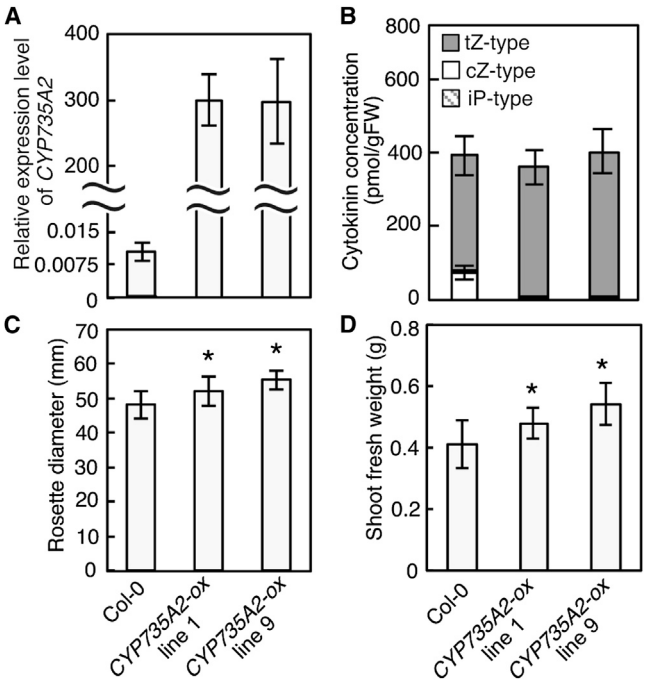



**Figure 5. Overexpression of *CYP735A2* Results in Shoot Growth Enhancement**

(A) Expression levels of *CYP735A2* in the shoots of wild-type (Col-0), *CYP735A2*-ox line 1, and line 9 grown for 21 days on soil. Quantitative RT-PCR analyses were conducted with cDNA generated from the plants indicated and specific primers. Expression levels were normalized using *ACT8* as an internal control.

(B–D) Cytokinin concentrations (B), rosette diameter (C), and fresh weight (D) of Col-0, *CYP735A2*-ox line 1 and line 9 shoots. Concentrations of each cytokinin species are shown in Table S4. Plants were grown for 3 weeks on soil and aerial parts were harvested. Error bars represent standard deviation of five biological replicates. Asterisks indicate statistically significant differences compared with Col-0 ($p < 0.05$, one-way ANOVA followed by Dunnett's test). gFW, gram fresh weight.

See also Figure S4.

between two CK compounds, iP and tZ. Although this mechanism appears to be unique among plant hormones, it can be speculated that similar mechanisms exist for other plant hormones including auxin and gibberellin, which exist in more than one active form (Hedden, 2001; Ludwig-Müller and Cohen, 2002; MacMillan, 2001; Simon and Petrasek, 2011; Yamaguchi, 2008).

## The *trans*-Hydroxylation of Cytokinins by *CYP735A*s in Plants

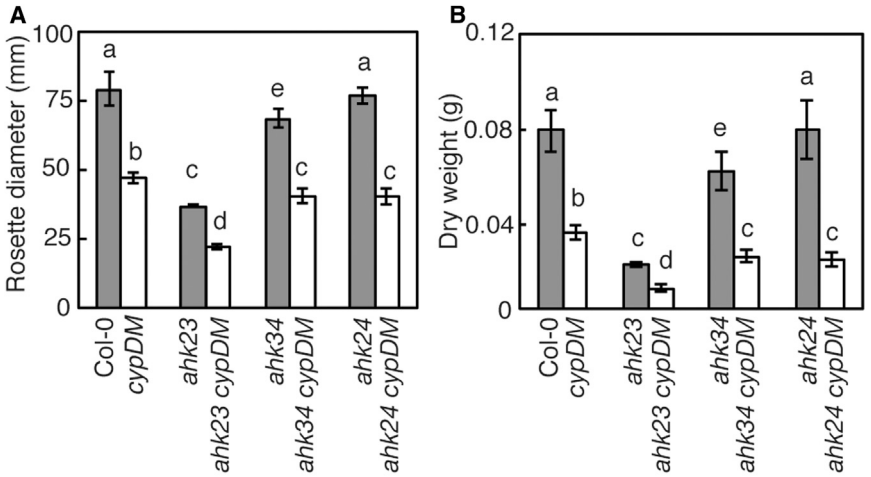
Although tZ was isolated as early as 1963 (Letham, 1963), the enzyme(s) responsible for the *trans*-hydroxylation of CKs remained unknown. Here we demonstrated that CYP735A1 and CYP735A2 catalyze the biosynthesis of tZ-type CKs in *Arabidopsis* (Figures 1 and S1; Table S1). However, the disruption of both genes does not result in the complete disappearance of tZ-type CKs (Table S1). Since *cyp735a1-1*, *cyp735a2-1*, and *cyp735a2-2* are null alleles (Figure S1B), additional minor pathway(s) for tZ-type CKs production probably exist. A possible pathway is the isomerization of cZ-type CKs, because *cis*-to-*trans* isomerase activity has been detected in kidney bean, potato, and rice (Bassil et al., 1993; Kudo et al., 2012; Suttle and Banowetz, 2000). It has been shown that cZ- and tZ-type CKs have side chains of different origin. The side-chain of tZ is mostly provided by the methylerythritol phosphate pathway, while that of cZ derives from the mevalonate pathway (Kasahara et al., 2004). A characterization of the origin of the tZ side chain in *cypDM* and further studies including the identification of gene(s) that encode *cis-trans* isomerase(s) are required to unravel the biological importance of the alternative pathway. In any case, our results reveal that CYP735A mediates the major pathway for tZ-type CK production in *Arabidopsis*.

*CYP735A* orthologs have been found in all seed plants whose genomes are completely sequenced. However, no *CYP735A* ortholog exists in *Selaginella moellendorffii*, *Physcomitrella patens*, *Chlamydomonas reinhardtii*, and *Volvox carteri* (Mizutani and Ohta, 2010; Nelson and Werck-Reichhart, 2011; http://www.phytozome.net), suggesting that *CYP735A* emerged after lycophytes and seed plants diverged. Interestingly, this coincides with the emergence of IPT (Frébort et al., 2011; Spíchal, 2012). It can be speculated that higher CK production and a diversification of CK function might have been required to control the elaborate developmental program of seed plants.

## The Role of *trans*-Hydroxylated Cytokinins in Shoots and Roots

In the vegetative stage, *cypDM* displays a retardation of shoot growth (Figures 2 and S2), which is similar to mutants that are deficient in CK biosynthesis, perception, or signaling (Argyros et al., 2008; Higuchi et al., 2004; Miyawaki et al., 2006; Nishimura et al., 2004; Riefler et al., 2006). On the other hand, during the reproductive stage, *cypDM* showed enhanced shoot branching (Figures 2G–2I and S2F; Table S2). Because such phenotype often is associated with increased CK levels (Chaudhury et al., 1993; McKenzie et al., 1998; Zubko et al., 2002), this raises the question whether all *cypDM* phenotypes result from diminished CK activity caused by tZ-deficiency. However, similar phenotypes have also been reported to be linked to low active CK levels in the shoots of overexpressors of *Arabidopsis CKX* and *LOG* genes (Kuroha et al., 2009; Werner et al., 2003). The level of tZ is more strongly affected than that of iP in these transgenic plants, suggesting that the relative amounts of tZ and iP might be relevant to these phenotypes. Alternatively, these phenotypes could be interpreted as results of attenuated SAM activity and concomitant reduction in apical dominance (Werner et al., 2003), although the shoot branching phenotypes of other CK-related mutants need to be scrutinized before general conclusions can be drawn.

Previous studies showed that root growth and development is usually enhanced in plants with reduced CK activity (Argyros et al., 2008; Dello Ioio et al., 2007; Higuchi et al., 2004; Miyawaki et al., 2006; Nishimura et al., 2004; Riefler et al., 2006; Werner et al., 2001, 2003; Yokoyama et al., 2007). In *cypDM* roots, however, neither an alteration in root growth and development (Figures 3A, 3B, and S3) nor a reduction in CK activity was observed (Figure 3C), suggesting that tZ is dispensable for normal root growth and development. Given that root growth is not affected in *CYP735A2* overexpressors (Figure S4), it appears to be regulated by CK quantity irrespective of CK type.




C

**Proportion of growth reduction by *cyp735a1 cyp735a2* mutations**

| Background | Col-0 | *ahk2 ahk3* | *ahk3 ahk4* | *ahk2 ahk4* |
|---|---|---|---|---|
| Dry weight (%) | 57 | 54 | 62 | 73 |
| Rosette diameter (%) | 41 | 40 | 41 | 48 |

**Figure 6. Shoot Growth of Plants Carrying Multiple Mutations in *cyp735a* and *ahk* Genes**

(A and B) Rosette diameter (A) and dry weight (B) of wild-type (Col-0), *ahk2-2 ahh3-3* (*ahk23*), *ahk3-3 cre1-12* (*ahk34*), *ahk2-2 cre1-12* (*ahk24*), *a1-2 a2-2* (*cypDM*), *ahk23 cypDM*, *ahk34 cypDM*, and *ahk24 cypDM*.

(C) Proportional growth reductions caused by the introduction of the *cyp735a1 cyp735a2* mutations into the Col-0, *ahk2 ahk3*, *ahk3 ahk4*, and *ahk2 ahk4* backgrounds. Values were calculated from data in (A) and (B). Plants were grown on soil for 24 days. Error bars represent SD of eight biological replicates. Different lowercase letters indicate statistically significant differences as indicated by Tukey's HSD test ($p < 0.05$).

It has been proposed that the differential ligand preferences of CK receptors are relevant to functional differences between iP and tZ (Higuchi et al., 2004; Nishimura et al., 2004; Romanov et al., 2006; Spíchal et al., 2004; Stolz et al., 2011). However, our studies of quadruple mutants produced by crossing *ahk* double mutants and *cypDM* suggest that the binding affinities of CK receptors might not be the only determinants of the functional differentiation between iP and tZ in the shoot (Figure 6). It is possible that iP and tZ are present in different tissues as in the case of phloem and xylem (Corbesier et al., 2003; Hirose et al., 2008; Kudo et al., 2010; Matsumoto-Kitano et al., 2008; Takei et al., 2001b). It is also possible that iP and tZ accumulate in different cellular compartments because several lines of evidence suggest that CK receptors are located in the endoplasmic reticulum (Caesar et al., 2011; Lomin et al., 2011, 2012; Wulfetange et al., 2011). Subcellular compartmentation of CKs was also proposed by Werner et al. (2003) based on the phenotypic differences between overexpressors of vacuole-targeted CKX and secreted CKX.

## *trans*-Hydoxylated Cytokinins as a Systemic Signal

CKs have been considered to act as long-distance signals because they are found in the phloem and xylem sap (Corbesier et al., 2003; Hirose et al., 2008; Kudo et al., 2010; Matsumoto-Kitano et al., 2008; Takei et al., 2001b). Recently Matsumoto-Kitano et al. (2008) clearly showed in grafting experiments using wild-type plants and the *ipt1;3;5;7* mutant that CKs transported between organs can regulate shoot as well as root growth. However, their experimental system did not allow them to assess the effects of iP- and tZ-type CK separately. Taking advantage of *cypDM*, we demonstrate that root-borne tZ-type CKs control shoot growth (Figure 7). Although the major site of tZ-type CK biosynthesis is in the root vasculature (Figure S5), the grafting experiments showed that root-borne tZ-type CKs are not necessarily required for normal shoot growth. Thus, the role of root-borne tZ-type CKs might be to stimulate shoot growth when roots sense favorable conditions. This notion is supported by the fact that an increment in tZ-type CKs alone can enhance shoot growth (Figure 5). A supply of a nitrogen source, for example nitrate, might be an example of such a situation. In this scenario, nitrate would trigger iP-type CK biosynthesis though the induction of *IPT3* (Miyawaki et al., 2004; Takei et al., 2004a), followed by CK-mediated upregulation of the expression of the *CYP735A*s (Takei et al., 2004b), finally leading to tZ-type CK accumulation in the root vasculature and transport to the shoot via the xylem. Such nitrogen-induced increases of tZ-type CK levels in the xylem have been reported from several species (Buban et al., 1978; Takei et al., 2001b; Wagner and Beck, 1993; Yong et al., 2000).

## The Side Chain of Cytokinin Carries a Signal

It has been shown that the side-chain structure of CKs is relevant to CK activity in bioassays (Leonard et al., 1969; Mok et al., 1978; Schmitz and Skoog, 1972; Sondheimer and Tzou, 1971), to the binding affinities of CK receptors (Choi et al., 2012; Jefferson et al., 1987; Romanov et al., 2006; Spíchal et al., 2004; Stolz et al., 2011; Yonekura-Sakakibara et al., 2004), and to the substrate specificity of CKX (Galuszka et al., 2007; Werner et al., 2001). This suggests that side-chain structure might carry a signal. However, the biological significance of side-chain variation has been controversial because side-chain structures are readily converted in plants (Bassil et al., 1993; Sondheimer and Tzou, 1971). By exploiting the first mutant impaired in CK side-chain modification, our study demonstrated that *trans*-hydroxylation of CK is a specific signal that facilitates growth in the shoot of *Arabidopsis*. The existence of *CYP735A* orthologs throughout the seed plants suggests that the signaling role of *trans*-hydroxylation might be highly conserved (Mizutani and Ohta, 2010; Nelson and Werck-Reichhart, 2011). Consistent with this idea, CK receptors with high affinity to tZ are found in maize (Lomin et al., 2011; Yonekura-Sakakibara et al., 2004). Thus, *CYP735A* appears a promising target for crop improvement because shoot growth enhancement could be achieved by increasing the expression of this gene without reducing root growth.

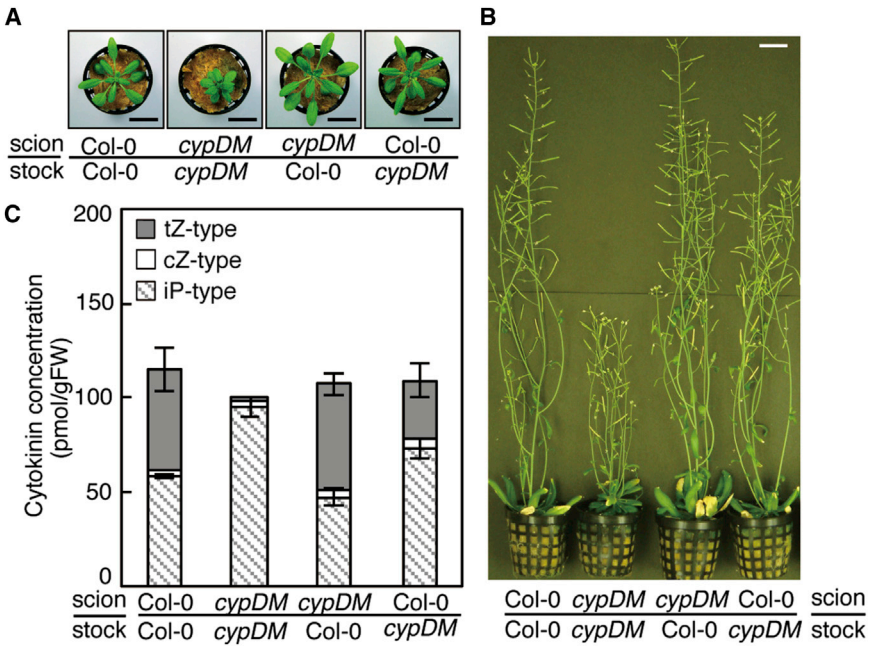



**Figure 7. Recovery of *cyp735a1 cyp735a2* Shoot Growth by Root-Borne tZ-Type Cytokinins**

(A and B) Three-day-old wild-type (Col-0) and *a1-2 a2-1* (*cypDM*) were grafted reciprocally by the wedge-grafting method and pictures were taken 38 days (A) and 58 days (B) after grafting.

(C) Cytokinin concentrations in rosette leaves (scions) of grafted plants at 45 days after germination. Error bars represent SD of four biological replicates. The concentration of each cytokinin species is shown in Table S5. gFW, gram fresh weight. Scale bar: 2 cm.

See also Figure S5.

## EXPERIMENTAL PROCEDURES

### Plant Material and Growth Conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild-type. The *cyp735a1-1* (SALK_063956), *cyp735A1-2* (SALK_093028), *cyp735a2-1* (SALK_077856), and *cyp735a2-2* (SALK_028195) lines were obtained from the Arabidopsis Biological Resource Center. For details of these lines, see the Supplemental Experimental Procedures. The *ipt3 ipt5 ipt7* triple mutant, and the cytokinin receptor mutants *ahk2-2*, *ahk3-3*, and *cre1-12*, were characterized previously (Higuchi et al., 2004; Miyawaki et al., 2006). Plants were grown on vertical agar plates, on rockfiber blocks (Nittobo), on soil (Metro-Mix 350, Sun Gro), or in hydroponic culture at 22°C under fluorescent light (100 μmol $m^{-2}$ $s^{-1}$, 16 hr light/8 hr dark).

### Quantification of Plant Hormones

Extraction and determination of the contents of cytokinins, auxin (IAA), and abscisic acid was performed as described previously (Kojima et al., 2009).

### Gene Expression Analysis

GUS staining was conducted as described elsewhere (Kiba et al., 2012). Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN). Total RNA was used for first strand cDNA synthesis by the SuperScript III First-Strand Synthesis System (Life Technologies) with oligo$(dT)_{20}$ primers, and subjected to semiquantitative and quantitative PCR.

### Complementation Analysis

Genomic fragments encompassing the putative promoter, coding region, and terminator of the *CYP735A* genes were amplified with specific primer sets gCYP735A1 and gCYP735A2, respectively (see Supplemental Experimental Procedures). The fragments were cloned into pBI101 (Clontech) in the place of the *GUS* gene and introduced into the *cyp735a1-1 cyp735a2-1* mutant.

To test the ability of externally applied cytokinins to complement the *cyp735a* double mutant phenotype, wild-type and *cyp735a1-2 cyp735a2-2* mutant seeds were sown on soil. Cytokinins were applied daily by spraying.

### Construction of *CYP735A2* Overexpression Lines and *Promoter:GUS* Reporter Lines

The *CYP735A2* cDNA was amplified with the specific primer set CYP735A2-ox (see Supplemental Experimental Procedures). The amplified fragment was cloned into pBI121 (Clontech) linked to the cauliflower mosaic virus (CaMV) 35S promoter, and introduced into wild-type *Arabidopsis* Col-0.

The *CYP735A1* and *CYP735A2* promoter regions were amplified with the specific primer sets ProCYP735A1 and ProCYP735A2, respectively. The fragments were introduced into the pBI101 binary vector (Clontech) to generate the *promoter:GUS* fusion genes, *ProCYP735A1:GUS* and *ProCYP735A2:GUS*. The fusion genes were introduced into wild-type *Arabidopsis* Col-0, generating ProCYP735A1:GUS and ProCYP735A2:GUS transgenic lines.

### Determination of Epidermal Cell Number and Rosette Diameter

For analysis of epidermal cells, soil-grown plants were used. The seventh rosette leaves were collected from 27-day-old plants and the adaxial side was scanned using a tabletop electron microscope TM3000 (Hitachi). To measure rosette diameter, plants were grown on soil and pictures were taken from above after indicated growth periods.

### Grafting

Micrografting of *Arabidopsis* seedlings was performed as described previously (Turnbull et al., 2002).

A more detailed version of the procedures is included in the Supplemental Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.10.004.

## ACKNOWLEDGMENTS

We are grateful to Tatsuo Kakimoto (Osaka University) for the *ipt3 ipt5 ipt7*, *ahk2-2*, *ahk3-3*, and *cre1-12* mutants. This work was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas (no. 21114005), a Grant-in-Aid for Young Scientists (B; no. 24780065), and the NC-CARP project from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The hormone analysis was supported by Japan Advanced Plant Science Network.

Received: May 15, 2013
Revised: September 12, 2013
Accepted: October 9, 2013
Published: November 25, 2013

## REFERENCES

Argyros, R.D., Mathews, D.E., Chiang, Y.H., Palmer, C.M., Thibault, D.M., Etheridge, N., Argyros, D.A., Mason, M.G., Kieber, J.J., and Schaller, G.E.

(2008). Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. Plant Cell *20*, 2102–2116.

Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmülling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. Plant Cell *23*, 69–80.

Bassil, N.V., Mok, D., and Mok, M.C. (1993). Partial purification of a *cis-trans*-isomerase of zeatin from immature seed of *Phaseolus vulgaris* L. Plant Physiol. *102*, 867–872.

Buban, T., Varga, A., Tromp, J., Knegt, E., and Bruinsma, J. (1978). Effects of ammonium and nitrate nutrition on levels of zeatin and amino nitrogen in xylem sap of apple rootstocks. Z Pflanzenphysiol *89*, 289–295.

Caesar, K., Thamm, A.M., Witthöft, J., Elgass, K., Huppenberger, P., Grefen, C., Horak, J., and Harter, K. (2011). Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. J. Exp. Bot. *62*, 5571–5580.

Chaudhury, A.M., Letham, S., Craig, S., and Dennis, E.S. (1993). *amp1* - a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative gowth, constitutive photomorphogenesis and precocious flowering. Plant J. *4*, 907–916.

Choi, J., Lee, J., Kim, K., Cho, M., Ryu, H., An, G., and Hwang, I. (2012). Functional identification of OsHk6 as a homotypic cytokinin receptor in rice with preferential affinity for iP. Plant Cell Physiol. *53*, 1334–1343.

Corbesier, L., Prinsen, E., Jacqmard, A., Lejeune, P., Van Onckelen, H., Périlleux, C., and Bernier, G. (2003). Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thaliana* during floral transition. J. Exp. Bot. *54*, 2511–2517.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and Sabatini, S. (2007). Cytokinins determine *Arabidopsis* root-meristem size by controlling cell differentiation. Curr. Biol. *17*, 678–682.

Foo, E., Morris, S.E., Parmenter, K., Young, N., Wang, H.T., Jones, A., Rameau, C., Turnbull, C.G.N., and Beveridge, C.A. (2007). Feedback regulation of xylem cytokinin content is conserved in pea and Arabidopsis. Plant Physiol. *143*, 1418–1428.

Frébort, I., Kowalska, M., Hluska, T., Frébortová, J., and Galuszka, P. (2011). Evolution of cytokinin biosynthesis and degradation. J. Exp. Bot. *62*, 2431–2452.

Galuszka, P., Popelková, H., Werner, T., Frébortová, J., Pospíšilová, H., Mik, V., Köllmer, I., Schmülling, T., and Frébort, I. (2007). Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. J. Plant Growth Regul. *26*, 255–267.

Gan, S., and Amasino, R.M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. Science *270*, 1986–1988.

Hedden, P. (2001). Gibberellin metabolism and its regulation. J. Plant Growth Regul. *20*, 317–318.

Higuchi, M., Pischke, M.S., Mähönen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., et al. (2004). *In planta* functions of the *Arabidopsis* cytokinin receptor family. Proc. Natl. Acad. Sci. USA *101*, 8821–8826.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., and Sakakibara, H. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. J. Exp. Bot. *59*, 75–83.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature *409*, 1060–1063.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. *6*, 3901–3907.

Kakimoto, T. (2001). Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. Plant Cell Physiol. *42*, 677–685.

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., Yamaguchi, S., and Sakakibara, H. (2004). Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in *Arabidopsis*. J. Biol. Chem. *279*, 14049–14054.

Kiba, T., Feria-Bourrellier, A.B., Lafouge, F., Lezhneva, L., Boutet-Mercey, S., Orsel, M., Bréhaut, V., Miller, A., Daniel-Vedele, F., Sakakibara, H., and Krapp, A. (2012). The *Arabidopsis* nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. Plant Cell *24*, 245–258.

Kojima, M., Kamada-Nobusada, T., Komatsu, H., Takei, K., Kuroha, T., Mizutani, M., Ashikari, M., Ueguchi-Tanaka, M., Matsuoka, M., Suzuki, K., and Sakakibara, H. (2009). Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography-tandem mass spectrometry: an application for hormone profiling in *Oryza sativa*. Plant Cell Physiol. *50*, 1201–1214.

Kudo, T., Kiba, T., and Sakakibara, H. (2010). Metabolism and long-distance translocation of cytokinins. J. Integr. Plant Biol. *52*, 53–60.

Kudo, T., Makita, N., Kojima, M., Tokunaga, H., and Sakakibara, H. (2012). Cytokinin activity of cis-zeatin and phenotypic alterations induced by overexpression of putative cis-Zeatin-O-glucosyltransferase in rice. Plant Physiol. *160*, 319–331.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature *445*, 652–655.

Kuroha, T., Tokunaga, H., Kojima, M., Ueda, N., Ishida, T., Nagawa, S., Fukuda, H., Sugimoto, K., and Sakakibara, H. (2009). Functional analyses of *LONELY GUY* cytokinin-activating enzymes reveal the importance of the direct activation pathway in *Arabidopsis*. Plant Cell *21*, 3152–3169.

Leonard, N.J., Hecht, S.M., Skoog, F., and Schmitz, R.Y. (1969). Cytokinins: synthesis, mass spectra, and biological activity of compounds related to zeatin. Proc. Natl. Acad. Sci. USA *63*, 175–182.

Letham, D.S. (1963). Zeatin, a factor inducing cell division isolated from *Zea mays*. Life Sci. *8*, 569–573.

Lomin, S.N., Yonekura-Sakakibara, K., Romanov, G.A., and Sakakibara, H. (2011). Ligand-binding properties and subcellular localization of maize cytokinin receptors. J. Exp. Bot. *62*, 5149–5159.

Lomin, S.N., Krivosheev, D.M., Steklov, M.Y., Osolodkin, D.I., and Romanov, G.A. (2012). Receptor properties and features of cytokinin signaling. Acta Naturae *4*, 31–45.

Ludwig-Müller, J., and Cohen, J.D. (2002). Identification and quantification of three active auxins in different tissues of *Tropaeolum majus*. Physiol. Plant. *115*, 320–329.

MacMillan, J. (2001). Occurrence of gibberellins in vascular plants, fungi, and bacteria. J. Plant Growth Regul. *20*, 387–442.

Matsumoto-Kitano, M., Kusumoto, T., Tarkowski, P., Kinoshita-Tsujimura, K., Václavíková, K., Miyawaki, K., and Kakimoto, T. (2008). Cytokinins are central regulators of cambial activity. Proc. Natl. Acad. Sci. USA *105*, 20027–20031.

McKenzie, M.J., Mett, V., Jameson, P.E., and Jameson, P.E.; Stewart Reynolds PH. (1998). Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. Plant Physiol. *116*, 969–977.

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. *37*, 128–138.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G., and Kakimoto, T. (2006). Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA *103*, 16598–16603.

Mizutani, M., and Ohta, D. (2010). Diversification of P450 genes during land plant evolution. Annu. Rev. Plant Biol. *61*, 291–315.

Mok, M.C. (1994). Cytokinins and plant development: An overview. In Cytokinins: Chemistry, Activity, and Function, F.D.W.S. Mok and M.C. Mok, eds. (Boca Raton, FL: CRC Press), pp. 155–166.

Mok, D.W., and Mok, M.C. (2001). Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. *52*, 89–118.

Mok, M.C., Mok, D.W., and Armstrong, D.J. (1978). Differential cytokinin structure-activity relationships in *phaseolus*. Plant Physiol. *61*, 72–75.

Nelson, D., and Werck-Reichhart, D. (2011). A P450-centric view of plant evolution. Plant J. *66*, 194–211.

Nieminen, K., Immanen, J., Laxell, M., Kauppinen, L., Tarkowski, P., Dolezal, K., Tähtiharju, S., Elo, A., Decourteix, M., Ljung, K., et al. (2008). Cytokinin signaling regulates cambial development in poplar. Proc. Natl. Acad. Sci. USA *105*, 20032–20037.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. Plant Cell *16*, 1365–1377.

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell *18*, 40–54.

Romanov, G.A., Lomin, S.N., and Schmülling, T. (2006). Biochemical characteristics and ligand-binding properties of *Arabidopsis* cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J. Exp. Bot. *57*, 4051–4058.

Sakakibara, H. (2006). Cytokinins: activity, biosynthesis, and translocation. Annu. Rev. Plant Biol. *57*, 431–449.

Schalk, M., Nedelkina, S., Schoch, G., Batard, Y., and Werck-Reichhart, D. (1999). Role of unusual amino acid residues in the proximal and distal heme regions of a plant P450, CYP73A1. Biochemistry *38*, 6093–6103.

Schmitz, R.Y., and Skoog, F. (1972). Cytokinins: synthesis and biological activity of geometric and position isomers of zeatin. Plant Physiol. *50*, 702–705.

Shaw, G. (1994). Chemistry of adenine cytokinins. In Cytokinins: Chemistry, Activity, and Function, D.W.S. Mok and M.C. Mok, eds. (Boca Raton, FL: CRC Press), pp. 15–34.

Simon, S., and Petrasek, J. (2011). Why plants need more than one type of auxin. Plant Sci. *180*, 454–460.

Sondheimer, E., and Tzou, D.S. (1971). The metabolism of hormones during seed germination and dormancy: II. The metabolism of 8-C-zeatin in bean axes. Plant Physiol. *47*, 516–520.

Spíchal, L. (2012). Cytokinins - recent news and views of evolutionally old molecules. Funct. Plant Biol. *39*, 267–284.

Spíchal, L., Rakova, N.Y., Riefler, M., Mizuno, T., Romanov, G.A., Strnad, M., and Schmülling, T. (2004). Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant Cell Physiol. *45*, 1299–1305.

Stolz, A., Riefler, M., Lomin, S.N., Achazi, K., Romanov, G.A., and Schmülling, T. (2011). The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. Plant J. *67*, 157–168.

Suttle, J.C., and Banowetz, G.M. (2000). Changes in *cis*-zeatin and *cis*-zeatin riboside levels and biological activity during potato tuber dormancy. Physiol. Plant. *109*, 68–74.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., and Mizuno, T. (2001). The Arabidopsis sensor His-kinase, AHk4, can respond to cytokinins. Plant Cell Physiol. *42*, 107–113.

Takei, K., Sakakibara, H., and Sugiyama, T. (2001a). Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. J. Biol. Chem. *276*, 26405–26410.

Takei, K., Sakakibara, H., Taniguchi, M., and Sugiyama, T. (2001b). Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. Plant Cell Physiol. *42*, 85–93.

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., Yamaya, T., and Sakakibara, H. (2004a). *AtIPT3* is a key determinant of nitrate-dependent cytokinin biosynthesis in *Arabidopsis*. Plant Cell Physiol. *45*, 1053–1062.

Takei, K., Yamaya, T., and Sakakibara, H. (2004b). *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-Zeatin. J. Biol. Chem. *279*, 41866–41872.

Tokunaga, H., Kojima, M., Kuroha, T., Ishida, T., Sugimoto, K., Kiba, T., and Sakakibara, H. (2012). Arabidopsis lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. Plant J. *69*, 355–365.

Turnbull, C.G., Booker, J.P., and Leyser, H.M. (2002). Micrografting techniques for testing long-distance signalling in *Arabidopsis*. Plant J. *32*, 255–262.

Wagner, B., and Beck, E. (1993). Cytokinins in the perennial herb *Urtica dioica* L. as influenced by its nitrogen status. Planta *190*, 511–518.

Walch-Liu, P., Neumann, G., Bangerth, F., and Engels, C. (2000). Rapid effects of nitrogen form on leaf morphogenesis in tobacco. J. Exp. Bot. *51*, 227–237.

Werner, T., Motyka, V., Strnad, M., and Schmülling, T. (2001). Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA *98*, 10487–10492.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell *15*, 2532–2550.

Werner, T., Nehnevajova, E., Köllmer, I., Novák, O., Strnad, M., Krämer, U., and Schmülling, T. (2010). Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. Plant Cell *22*, 3905–3920.

Wulfetange, K., Lomin, S.N., Romanov, G.A., Stolz, A., Heyl, A., and Schmülling, T. (2011). The cytokinin receptors of Arabidopsis are located mainly to the endoplasmic reticulum. Plant Physiol. *156*, 1808–1818.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., and Mizuno, T. (2001). The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol. *42*, 1017–1023.

Yamaguchi, S. (2008). Gibberellin metabolism and its regulation. Annu. Rev. Plant Biol. *59*, 225–251.

Yokoyama, A., Yamashino, T., Amano, Y., Tajima, Y., Imamura, A., Sakakibara, H., and Mizuno, T. (2007). Type-B ARR transcription factors, ARR10 and ARR12, are implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of *Arabidopsis thaliana*. Plant Cell Physiol. *48*, 84–96.

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T., and Sakakibara, H. (2004). Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to cis-zeatin. Plant Physiol. *134*, 1654–1661.

Yong, J.W., Wong, S.C., Letham, D.S., Hocart, C.H., and Farquhar, G.D. (2000). Effects of elevated [CO(2)] and nitrogen nutrition on cytokinins in the xylem sap and leaves of cotton. Plant Physiol. *124*, 767–780.

Zubko, E., Adams, C.J., Macháèková, I., Malbeck, J., Scollan, C., and Meyer, P. (2002). Activation tagging identifies a gene from *Petunia hybrida* responsible for the production of active cytokinins in plants. Plant J. *29*, 797–808.